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Communication to the Editor

Insecticide Resistance in a Strain of *Aphis gossypii* from Southern France

Robert Delorme,* Danielle Augé, Marie-Thérèse Béthenod & François Villatte

Unité de Phytopharmacie et Médiateurs chimiques, INRA, Route de St Cyr, 78026 Versailles Cedex, France

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Abstract: A strain (R) of *Aphis gossypii* from Southern France was found to be resistant to several insecticides, particularly to pirimicarb, as compared to a susceptible strain (S). Resistance levels were determined by biological tests, and the highest resistance factor (1350) was for pirimicarb. Resistance was mainly restricted to anticholinesterase inhibitors. Use of synergists, DEF and PB, suggested that resistance mechanisms based on detoxification were involved to a minor extent, since a good correlation was observed between I_{50} values and k_i values of AChE and *in-vivo* bioassay data. The two strains differed in esterase activity, with a 27·7-fold increase in the R strain. Resolution of esterases by polyacrylamide gel electrophoresis showed different patterns in the S and R strains, and two isozymes were less sensitive to pirimicarb in the S strain; however, no *in-vitro* degradation of [^{14}C]pirimicarb was observed. These data suggest that the main mechanism of resistance was through a decrease in the sensitivity of the target, AChE, to the insecticides.

Key words: *Aphis gossypii*, resistance, pirimicarb, acetylcholinesterase, carboxylesterases

1 INTRODUCTION

The cotton or melon aphid *Aphis gossypii* (Glover) is a highly polyphagous pest able to develop on a broad range of host plants, with a preference for cucurbits,[1,2] where it causes serious damage.[3] In cotton, this pest can decrease yield and quality.[4] Chemical protection has led to resistance of *A. gossypii* in many countries. The first report concerned pirimicarb, twenty years ago in England in chrysanthemum crops.[5] Resistance to organophosphates and carbamates has been reported in the Soviet Union,[6] China,[7] Israel,[8] Japan,[9,10] UK,[11,12] Hawaii[13] and the USA.[14,15] Pyrethroid resistance was mentioned in China,[16] Israel[8] and the USA.[14] On cotton crops in Sudan, this aphid is resistant to all the main insecticide groups.[17] Mechanisms underlying these resistances are varied: acetylcholinesterase (AChE) insensitivity,[5,17–20] enhancement of carboxylesterases[7,9,15] and enhanced oxidative metabolism.[7,21] In France, some failures of pirimicarb were reported in 1987 on melon, and resistance was confirmed on a strain collected at St Martin-de-Crau (Provence).[18] The aims of the present work were to study the resistance to insecticides of a resistant strain of *A. gossypii* from Southern France in comparison with a susceptible reference strain and to determine the underlying biochemical mechanisms.

2 MATERIALS AND METHODS

2.1 Aphid populations

The resistant strain (R) was collected in 1992 at Pernes-les-Fontaines (Provence) from a sweet pepper (*Capsidum*

* To whom correspondence should be addressed.

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annuum L.) field. A susceptible clone (S) was obtained from Dr G. Labonne (INRA-ENSA Montpellier) who collected it in 1985 at Navacelles (Provence) in a non-treated area. The R and S strains were kept and reared anholocyclically on cucumber seedlings (*Cucumis sativus* L., CV. Marketer), in controlled-environment chambers with a temperature of 20(±1)°C, 70(±10)% relative humidity and a 16 : 8 h light : dark photoperiod. No selection pressure on the R strain has been applied since the strain was collected in 1992 and the susceptibility and resistance level of each strain was measured by bioassays performed twice per year.

2.2 Bioassays

Sixteen insecticides were tested for contact toxicity (Table 1). Formulations were gifts from commercial sources. The choice of compounds was based on their practical use and their chemical structures. The procedure was the same as described previously.[17] Tests were repeated three times and resistance factors (RF) were determined as the LC_{50} of the R strain/LC_{50} of the S strain.

2.3 Synergism

The action of two synergists was investigated to determine whether metabolism was involved in resistance. The synergists were piperonyl butoxide (PB), an MFO (Mixed Function Oxidase) inhibitor and *S*,*S*,*S*-tributyl phosphorotrithioate (DEF), an inhibitor of both esterases and glutathione transferases. They were applied 1 h prior to insecticide application by spraying as described previously for the insecticides. Doses (1000 mg litre^{-1} for PB and 200 mg litre^{-1} for DEF) were the highest concentration inducing no observable toxicity on the S and R strains. Synergism ratios (LC_{50} insecticide alone/LC_{50} insecticide + synergist) were calculated for both strains.

2.4 Acetylcholinesterase assay

Among insecticides tested *in vivo*, five AChE inhibitors were studied for I_{50} and k_i: paraoxon-ethyl, (*E*)-mevinphos, methomyl, pirimicarb and triazamate. All were technical grade of the highest purity available and were gifts from industrial producers. Paraoxon-ethyl was used instead of parathion-ethyl, the thiol analogue

TABLE 1
LC_{50} of Insecticides against Two Strains of *Aphis gossypii* and Resistance Factors

Compounds	*LC_{50} (g hl^{-1}) (95% confidence limits)*		*Slope*		
	S strain	*R strain*	*S strain*	*R strain*	*RF*[a]
Organochlorines					
Endosulfan	2·07 [1·93–2·23]	2·30 [2·15–2·45]	3·35	4·64	1·11
γ-BHC	4·57 [3·97–5·27]	3·52 [3·22–3.84]	2·30	3·17	0·770
Organophosphates					
Acephate	1·95 [1·79–2·11]	9·36 [8·12–10·8]	3·58	3·77	4·80
Dimethoate	0·228 [0·199–0·262]	18·0 [15·7–20·6]	3·53	2·29*	78·9
Ethyl-parathion	0·694 [0·632–0·763]	7·57 [6·82–8·41]	4·35	3·62	10·9
Methidation	0·471 [0·406–0·547]	1·60 [1·50–1·72]	4·16	4·85	3·40
Mevinphos	0·343 [0·316–0·373]	0·882 [0·803–0·968]	4·91	5·00	2·57
Carbamates					
Methomyl	0·383 [0·325–0·451]	1·13 [1·02–1·26]	1·96	2·06	2·95
Pirimicarb	0·152 [0·142–0·163]	205 [156–270]	4·61	1·87*	1350
Pyrethroids					
Bifenthrin	0·003 32 [0·002 82–0·003 91]	0·006 41 [0·005 71–0·007 20]	2·25	2·20	1·93
Deltamethrin	0·0107 [0·009 53–0·0120]	0·0239 [0·0211–0·0271]	2·22	2·37	2·23
Fenvalerate	0·140 [0·120–0·164]	0·119 [0·107–0·134]	1·62	2·06	0·850
Lambda-cyhalothrin	0·004 09 [0·003 71–0·004 52]	0·004 10 [0·003 40–0·004 95]	3·16	1·97*	1·00
Tau-Fluvalinate	0·740 [0·0650–0·0843]	0·0915 [0·0800–0·105]	2·18	2·06	1·24
Chlornicotinyles					
Imidacloprid	0·165 [0·151–0·180]	0·168 [0·151–0·188]	2·67	2·54	1·02
Triazoles					
Triazamate	0·168 [0·159–0·177]	5·49 [4·91–6·14]	4·99	4·78	32·7

[a] RF = LC_{50} R strain/LC_{50} S strain (mean of three replicates).
[b] * Significantly different from S strain (Student test, $P = 0·05$).

that is a poor inhibitor *in vitro*. We could not determine the I_{50} or k_i for dimethoate because the I_{50} was greater than 10^{-3} M and dimethoxon was not available. (*E*)-Mevinphos was chosen because it is the more potent isomer. AChE activity was measured by the Ellman method[22] using 10^{-5} M DTNB. Ten apterous adults were homogenised in ice-cold phosphate buffer (1 ml; 25 mM, pH 7) containing 'Triton' X-100 (1·0 ml litre^{-1}) in an Eppendorf tube with a Teflon pestle and centrifuged at 10 000*g* at 4°C for 7 min. The supernatant was the enzyme source. For the determination of anticholinesterase activity, phosphate buffer (870 μl) homogenate (100 μl) and the appropriate amount of inhibitor dissolved in acetone (10 μl) were preincubated for 5 min at 25°C. In the control tubes, the inhibitor was replaced by acetone and in the blank by a 10^{-3} M eserine acetone + methanol (90 + 10 by volume). Acetylthiocholine (10 μl; 100 mM) and DTNB (10 μl, 1 mM) were added and tubes were incubated for 5 min at 25°C. The absorbance was measured by a Hitachi U1100 spectrophotometer at 412 nm. The I_{50} value was calculated from five inhibitor concentrations by computed linear regression. For determination of bimolecular rate constants, k_i, insecticide was incubated with homogenate at a concentration equal to the I_{50} at 25°C. Aliquots were taken and residual activity of AChE was recorded over time by the method described above, the slope was calculated by linear regression on a microcomputer and the k_i value was calculated as described by Main and Iverson.[23]

2.5 Carboxylesterase assay

The carboxylesterase activity was measured colorimetrically as described by Devonshire.[24] Individual aphids were weighed on a Sartorius micro-balance and homogenised in phosphate buffer (1 ml; 0·4 M, pH 7). Sixty microlitres of the homogenate were used for the assay. Under these conditions, increase in colour density was proportional to enzyme activity and measured at 605 nm on a U1100 Hitachi spectrophotometer.

2.6 Polyacrylamide gel electrophoresis

Electrophoresis was performed using a vertical Bio-Rad electrophoresis unit with a 7·5% acrylamide gel. Aphids were homogenised in Tris HCl buffer (pH 8·8) containing 'Triton' X-100 (1·0 ml litre^{-1}): 300 μl buffer mg^{-1} aphid for the R strain and 20 μl buffer mg^{-1} aphid for the S strain. The homogenate was centrifuged at 15 000*g* for 10 min at 4°C. Samples of supernatant were electrophoresed for 2 h at 200 V in a continuous system at 4°C, using Tris glycine (pH 8·8) as the buffer. Total esterases were strained for 20 min with shaking in 2 ml Tris HCl, 94 ml water, 50 mg Fast Blue RR and 2 ml of 10 g litre^{-1} α-naphthyl acetate in acetone. For the inhibition assay, gels were preincubated at 4°C in a Tris KCl buffer (0·2 M) containing pirimicarb ($1{\cdot}7 \times 10^{-4}$ M) for 30 min.

2.7 *In-vitro* metabolism of [^{14}C]pirimicarb

[^{14}C]Pirimicarb (specific activity 2·044 GBq mmol^{-1}) was provided by Zeneca Agrochemicals Company. Enzyme was prepared by homogenising 60 mg aphids in ice-cold phosphate buffer (2 ml; 50 mM, pH 7·2), containing EDTA, 1; DTT 0·1 and PMSF 0·4 mM, in a Potter homogeniser. Homogenates were centrifuged at 12 000*g* for 10 min at 4°C. Supernatant (1 ml) was incubated with an acetonic [^{14}C]pirimicarb solution (10 μl; final concentration 10^{-5} M, $1{\cdot}2 \times 10^6$ dpm) and an aqueous NADPH solution (26 μl; 0·04 M). After incubation for 24 h in the dark at 25°C, 50 μl were spotted on the TLC plater (Merck 60 SiO_2) and chromatographed with hexane + ethyl acetate + methanol (5 + 4 + 1 by volume). Results were obtained by direct counting with a linear counter (Bertold) and autoradiography (Hyperfilm β Max) for seven days at − 10°C. Other measurements were obtained by HPLC (reverse phase), coupled with a solid scintillation counter.

3 RESULTS

3.1 Bioassays

Table 1 summarises the toxicological data for the S and R strains for the 16 insecticides tested. The susceptible clone from Navacelles was killed at low doses of the insecticides tested, with the exception of γ-BHC, which is considered an inadequate aphicide. This high susceptibility confirms the value of the Navacelles clone as a reference strain. The R strain from Pernes-les-Fontaines possessed a high level of resistance to pirimicarb (RF = 1350) and a weaker resistance to the other carbamate methomyl (RF = 2·95). Resistance factors for organophosphorus compounds ranged from 2·57 for mevinphos to 78·9 for dimethoate. Moderate (RF = 33) resistance was also found for another AChE inhibitor, triazamate. No resistance was found to organochlorines, imidacloprid or pyrethroids. Only a small significant difference was seen between the S and R strains for bifenthrin and deltamethrin. Since resistance seemed to be restricted to compounds acting as anticholinesterase inhibitors, these data suggested an insensitive AChE in the R strain, although this insensitivity was clearly not expressed to all inhibitors.

3.2 Synergism experiments

Synergism experiments were conducted with the two insecticides having the highest resistance factors in the R strain: pirimicarb and dimethoate. PB reduced the

TABLE 2
Toxicity of Insecticides tested against S and R Strains of *Aphis gossypii* in Absence and in Presence of PB or DEF. Synergism Ratio (SR) and Resistance Factor (RF) in Absence and in Presence of Synergist

	S strain		*R strain*		
Product	LC_{50} ($g\ hl^{-1}$) (*95% CL*)	*SR*	LC_{50} ($g\ hl^{-1}$) (*95% CL*)	*SR*	*RF*
Pyrimicarb	0·152 (0·142–0·163)		205 (156–270)		1350
+PB	0·0176 (0·0153–0·0203)	8·64	29·1 (26·7–31·8)	7·04	1650
+DEF	0·0465 (0·0350–0·0619)	3·27	26·3 (23·3–29·8)	7·79	566
Dimethoate	0·228 (0·199–0·262)		18·0 (15·7–20·6)		78·9
+PB	0·155 (0·138–0·174)	1·47	12·1 (11·3–12·9)	1·49	78·1
+DEF	0·174 (0·158–0·191)	1·31	4·70 (4·45–4·97)	3·83	27·0

LC_{50} of pirimicarb by a synergism ratio (SR) of 8·64 and 7·04 in the S and R strains respectively (Table 2). For dimethoate, the SR values were, respectively, 1·47 and 1·49 for the S and R strains. This suggests that MFOs were probably involved in the metabolism of at least pirimicarb. As the SR values were not different for the two strains, MFOs seemed not to be involved as a mechanism of resistance. DEF reduced the LC_{50} of pirimicarb, with SR values 3·27 and 7·80 for the S and R strains, respectively. For dimethoate the SR values were respectively 1·31 and 3·83 for the S and R strains. DEF synergised 2- to 3-fold more pirimicarb and dimethoate

TABLE 3
Anticholinesterase Activity of Five Insecticides for the S and R Strains of *Aphis gossypii*

Parameter[a]*/pesticide*	*S strain*	*R strain*	*Ratio R/S*
I_{50} (M) (95% CL)			
Paraoxon-ethyl	$5{\cdot}52\ (2{\cdot}10–14{\cdot}50) \times 10^{-7}$	$2{\cdot}55\ (2{\cdot}24–2{\cdot}91) \times 10^{-6}$	4·62
(*E*)-Mevinphos	$3{\cdot}71\ (2{\cdot}64–5{\cdot}19) \times 10^{-9}$	$7{\cdot}18\ (5{\cdot}52–9{\cdot}34) \times 10^{-8}$	19·4
Methomyl	$4{\cdot}51\ (2{\cdot}92–6{\cdot}94) \times 10^{-6}$	$2{\cdot}91\ (2{\cdot}02–4{\cdot}16) \times 10^{-6}$	0·645
Pirimicarb	$1{\cdot}01\ (0{\cdot}501–2{\cdot}02) \times 10^{-6}$	$2{\cdot}13\ (1{\cdot}72–2{\cdot}64) \times 10^{-4}$	210
Triazamate	$9{\cdot}66\ (8{\cdot}55–10{\cdot}92) \times 10^{-8}$	$4{\cdot}95\ (3{\cdot}55–6{\cdot}91) \times 10^{-7}$	5·12
k_i ($M^{-1}\ min^{-1}$)(±SE)			
Paraoxon-ethyl.	$3{\cdot}04\ (\pm 0{\cdot}20) \times 10^{5}$	$4{\cdot}81\ (\pm 0{\cdot}26) \times 10^{4}$	6·32
(*E*)-Mevinphos	$6{\cdot}45\ (\pm 0{\cdot}64) \times 10^{7}$	$2{\cdot}59\ (\pm 0{\cdot}15) \times 10^{6}$	24·9
Methomyl	$2{\cdot}42\ (\pm 0{\cdot}16) \times 10^{4}$	$1{\cdot}75\ (\pm 0{\cdot}63) \times 10^{4}$	1·32
Pirimicarb	$3{\cdot}07\ (\pm 0{\cdot}20) \times 10^{5}$	$2{\cdot}55\ (\pm 0{\cdot}10) \times 10^{2}$	1200
Triazamate	$1{\cdot}57\ (\pm 0{\cdot}20) \times 10^{6}$	$2{\cdot}36\ (\pm 0{\cdot}76) \times 10^{5}$	6·65

[a] Inhibitory concentration for 50% of AChE activity (I_{50}). Bimolecular rate constant (k_i). Means of three replicates.

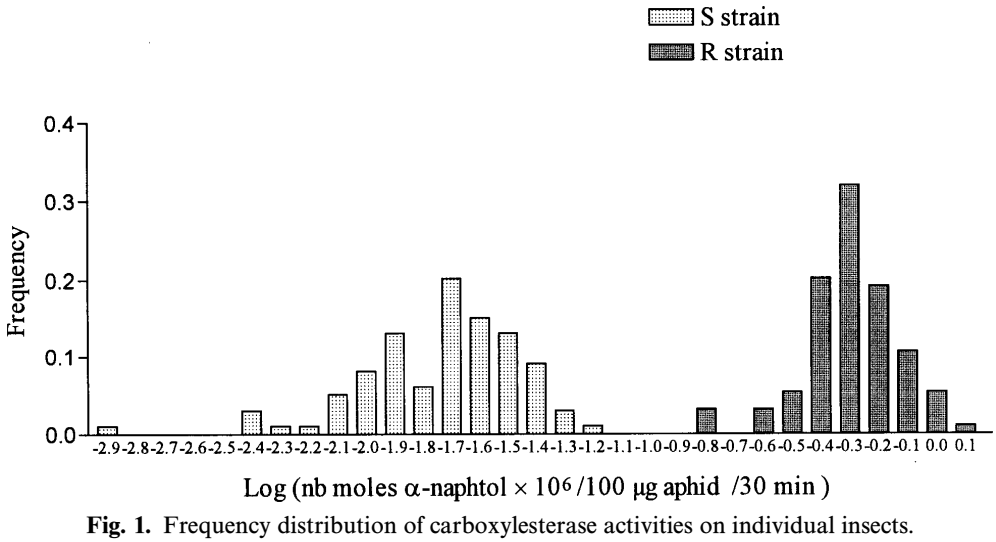

Fig. 1. Frequency distribution of carboxylesterase activities on individual insects.

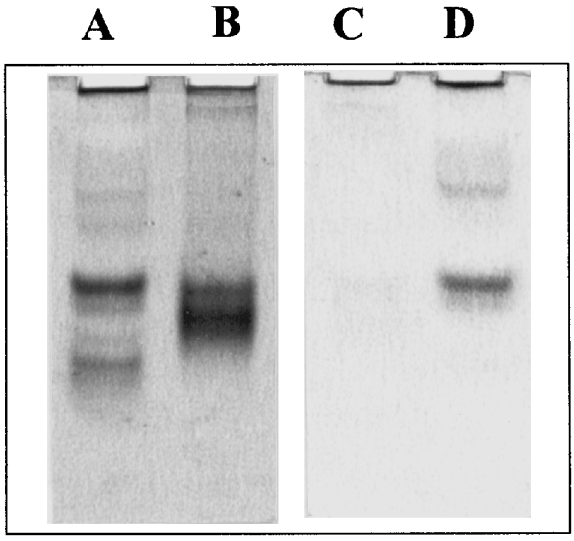

Fig. 2. Native electrophoresis of general esterases (2 h, 200 V), with Fast Blue RR staining. A: S strain, B: R strain (15-fold diluted), C: R strain (15-fold diluted) after 30 min incubation with pirimicarb $1{\cdot}7 \times 10^{-4}$ M, D: S strain after 30 min incubation with pirimicarb $1{\cdot}7 \times 10^{-4}$ M.

in the R strain than in the S strain. This could indicate that carboxylesterases played a minor role in resistance.

3.3 AChE assay

The method used to determine I_{50} did not involve any dilution of the insecticide after addition of substrate, but no inhibition was noticed during the reaction time (linear reaction). The I_{50} values and *in-vivo* toxicological data seemed to be correlated (Table 3), with the exception of mevinphos. The I_{50} R/I_{50} S ratio for pirimicarb was 210 and the *in-vivo* resistance factor (RF) was 1350. For paraoxon-ethyl and triazamate, the I_{50} value for the R strain showed a 5-fold increase, which corresponded to RF values of 10·9 and 32·7 respectively. No significant difference was found for methomyl (RF = 2·95). On the other hand, the 19·4-fold increase of the I_{50} value for (*E*)-mevinphos was not correlated with a high level of resistance. These results suggested that decrease in the sensitivity of the target was the main mechanism underlying resistance.

The calculation of k_i, which is a measure of the overall inhibitor potency of a compound, was possible due to the linearity of the metabolism curves. For pirimicarb, there were 1200 units of difference between the R and S strains, possibly reflecting a change in the catalytic centre of the enzyme.

3.4 Frequency distributions of carboxylesterase activities on individuals

Distributions of individual carboxylesterase activities showed no overlap between the S and R strains (Fig. 1). When activities were expressed as their log, the distributions could be fitted to Gaussian curves with a high correlation factor (0·87 and 0·97 for S and R strains, respectively) demonstrating the homogeneity of the strains. The ratio between the mean activities within the

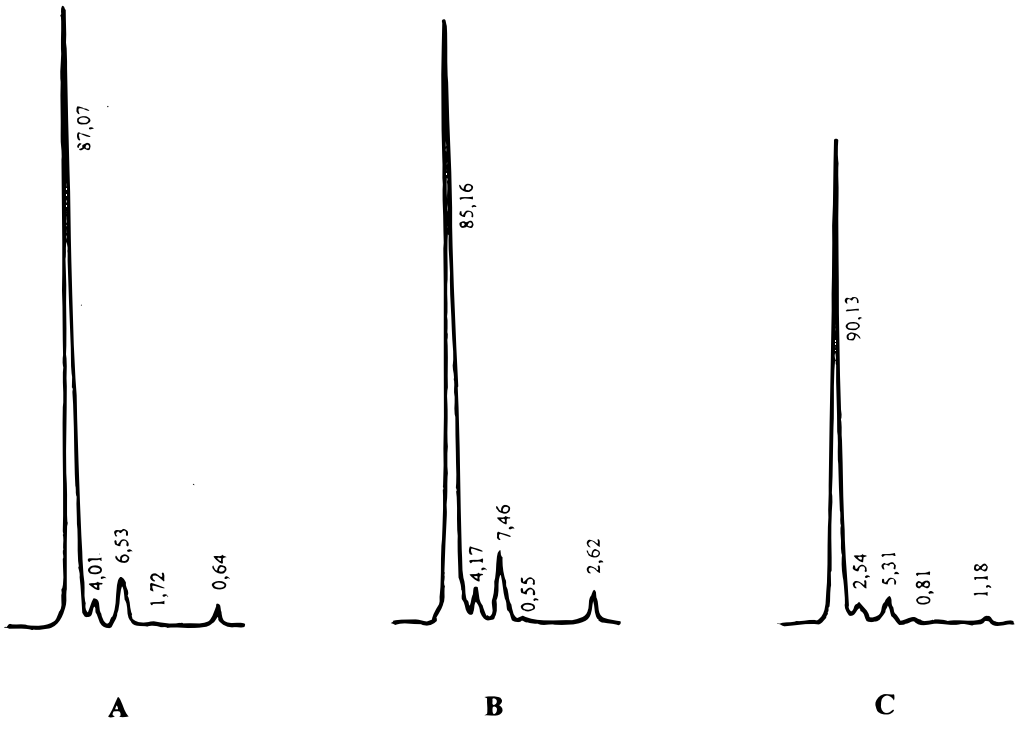

Fig. 3. *In-vitro* degradation of [^{14}C]pirimicarb. Quantification by linear counting of radioactivity on TLC. A: S strain, B: R strain, C: reference (homogenate replaced by phosphate buffer).

two strains ($1{\cdot}99 \times 10^{-8}$ mole 100 μg^{-1} 30 min^{-1} and $5{\cdot}52 \times 10^{-7}$ mole 100 μg^{-1} 30 min^{-1} for S and R strains respectively) was 27·7.

3.5 Native PAGE

The electrophoretic patterns of the esterases from S and R strains were different (Fig. 2), indicating a difference in the electrophoretic mobility of the enzymes, i.e. qualitative differences in the esterases from the two strains. These electrophoretic patterns were similar to those observed in *A. gossypii* by other authors.[5,9] Homogenate of R strain was diluted 15-fold in order to improve band resolution and confirm the quantitative changes mentioned previously. Inhibition assay by pirimicarb revealed differences in the sensitivities of two isozymes. All bands were inhibited in the R strain, whereas two bands remained visible in the S strain.

3.6 *In-vitro* degradation of [^{14}C]pirimicarb

Quantification obtained by direct counting (Fig. 3) as well with HPLC and autoradiography (results not shown) failed to show any noticeable differences between the control, the S strain and the R strain. The major peak corresponds to [^{14}C]pirimicarb and the minor ones to impurities present in the initial solution. To check for a weak metabolism masked by too much [^{14}C]pirimicarb, the experiment was repeated with ten-fold less substrate. Results were similar. A third experiment using the same conditions as for the carboxylesterase assay (phosphate buffer pH 7, 25 mM) gave the same results. Thus, in our conditions, we did not observe any noticeable breakdown of pirimicarb *in vitro*.

4 DISCUSSION

The bioassay in *A. gossypii* showed that the R strain from Pernes-les-Fontaines was highly resistant to pirimicarb, the main insecticide used in Southern France against *A. gossypii*. Similarly to observations by others authors,[5] it was found that resistance to pirimicarb was stable in the absence of selection pressure, with an RF that was essentially unchanged over several years of rearing in captivity. Probit plots issued from bioassays and data from I_{50} determination experiments suggested that the R strain is homogenous. Slopes of the concentration–mortality lines were significantly lower in the R strain concerning pirimicarb, dimethoate and lambda cyhalothrin. In general, the slope is correlated with genetic variability, but some authors have recently shown that this linkage is doubtful.[25] The resistance level to pirimicarb was not found for the other carbamate insecticides tested; nevertheless, lower resistance was detected to some organophosphorus compounds (dimethoate, parathion-ethyl) and triazamate. All these insecticides are acetylcholinesterase inhibitors. The correlation observed in the AChE assay between the I_{50} value and the *in-vivo* bioassay points to a decrease in the sensitivity of the target as the main mechanism underlying resistance. The slight resistance to mevinphos, opposed to the higher insensitivity of the AChE from the R strain, could be due to the presence of the two isomers in the commercial formulation used in bioassays, whereas only the (*E*) isomer has been tested in AChE inhibition experiments. Synergist experiments using a combination of pirimicarb and dimethoate on one hand, PBO and DEF on the other hand, suggested that resistance mechanisms based on detoxification were only very slightly involved in resistance. The slight effect of DEF, an esterase inhibitor, on the resistance factor for both pirimicarb and dimethoate led us to study the carboxylesterase activity of the S and R strains.

Surprisingly, we found important quantitative and qualitative differences between the two strains. Quantitatively, the ratio for carboxylesterase activity was 27·7 and the level of activity for R strain was of the same order of magnitude as for *Myzus persicae* resistant clones,[24,26] in which carboxylesterases are known to be responsible for resistance against some OP and carbamates. Moreover, electrophoretic patterns of esterases showed bands with important differences in electrophoretic mobilities between the two strains. These patterns were similar to those obtained by other authors.[5,9] We have no precise idea of the possible change(s) leading to these differences, but we could suggest an esterase glycosylation in the R strain, as observed on a resistant strain of *Laodelphax striatellus* (Fall.).[27] In our case, the susceptibility of the two isozymes to pirimicarb was different between the two strains: two bands remained visible in the S strain after incubation with the insecticide, whereas all bands disappeared in the R strain. Esterase modifications in resistant strains of *A. gossypii* have been noted by several authors[15,28] and have been claimed to be responsible for resistance, but no other mechanisms have been investigated. Thus, in our case, despite esterasic changes, no noticeable differences were found in *in-vitro* degradation of [^{14}C]pirimicarb, even in optimal conditions for esterases. In consequence, we could not establish a link between esterase changes and resistance, since the hypothesis of a phylogenetic inter-strain variation could not be definitively excluded.

The above combined data suggested a minor incidence of carboxylesterases in the resistance to the studied compounds, acting probably by a weak sequestration of toxicants, as observed for other species.[24,29] Thus, the main mechanism of resistance in this strain of

A. gossypii was the modification(s) of the target, as is the case in an increasing number of species. Characterisation of AChE in this aphid, and determination of the different alleles in natural populations may contribute to a better knowledge of its resistance status and provide tools for an improved monitoring and management.

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